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(54) Title: CELL SURFACE PROTEIN PRESENT ON NK (NATURAL KILLER CELLS) NAMED DX1

(57) Abstract

NK cell surface antigen from a mammal, reagents related thereto including purified proteins, specific antibodies, and nucleic acids encoding said antigen. Methods of using said reagents and diagnostic kits are also provided.

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CELL SURFACE PROTEIN PRESENT ON NK (NATURAL KILLER CELLS) NAMED DX1

BACKGROUND OF THE INVENTION

The immune system of vertebrates consists of a number of organs and several different cell types. Two major cell types include the myeloid and lymphoid lineages. Among the lymphoid cell lineage are B cells, which were originally characterized as differentiating in fetal liver or adult bone marrow, T cells, which were originally characterized as differentiating in the thymus, and natural killer (NK) cells. See, e.g., Paul (ed.) (1993) Fundamental Immunology (3d ed.) Raven Press, New York.

NK cells are lymphocytes that mediate cytotoxicity and secrete cytokines after immune stimulation. NK cells have been implicated in immunity against certain viruses, intracellular bacteria, and parasites. There is also evidence for NK cell involvement in rejection of allogeneic murine hematopoietic cells and in the recognition and lysis of normal and malignant cells lacking MHC class I antigens.

In many aspects of the development of an immune response or cellular differentiation, soluble proteins, e.g., cytokines, and cell surface antigens, e.g., CD markers, play critical roles in regulating cellular interactions. These cytokines and cell markers apparently mediate cellular activities in many ways. They have been shown, in many cases, to modulate proliferation, growth, and differentiation of hematopoietic stem cells into the vast number of progenitors composing the lineages responsible for an immune response.

However, the cellular molecules which are expressed by different developmental stages of cells in these maturation pathways are still incompletely identified. Moreover, the roles and mechanisms of action of signaling molecules which induce, sustain, or modulate the various physiological, developmental, or proliferative states of these cells is poorly understood.

While the receptors used by NK cells for antigen recognition have remained elusive, recent studies indicate that certain genes of the C-type lectin superfamily are expressed by NK cells and may be involved in NK cell function.

Two distinct families of related genes within the C-type lectin superfamily, designated NKR-P1 and Ly-49, have been identified on mouse chromosome 6 that encode proteins preferentially expression on NK cells. At present, at least 5 murine Ly-49 genes have been identified. The proteins encoded by Ly-49 genes are type II membrane glycoproteins that form disulfide-bonded homodimers. NKR-P1 was identified initially by the 3.2.3 mAb which recognizes a disulfide-linked homodimer expressed on rat NK cells, granulocytes and a minor subset of T cells. At least 3 mouse genes have been cloned that are homologous to rat NKR-P1. The prototype murine NK marker, NK1.1, is encoded by one of the mouse NKR-P1 genes. Cross-linking NKR-P1 glycoproteins on the surface of rodent NK cells triggers NK cell-mediated cytotoxicity, secretion of cytokines, and IP_3 generation.

The rodent NKR-P1 and human DX1 genes, as members of the C-type lectin superfamily, have several characteristically conserved structural properties. Of note is the WIGL motif that is conserved in the carbohydrate recognition domain of all C-type lectins. The C-type lectin superfamily also includes the NK-cell associated antigens Ly-49 and NKG2 and the early activation antigen CD69. However, the homology of DX1 with human NKG2A is only 26% and the similarity with murine Ly49 and human CD69 is even less.

Clearly, the immune system and its response to various stresses have relevance to medicine, e.g., infectious diseases, cancer related responses and treatment, allergic and transplantation rejection responses. See, e.g., Thorn, et al. Harrison's Principles of Internal Medicine McGraw/Hill, New York. Medical science relies, in large degree, to appropriate recruitment or suppression of the immune system in effecting cures for insufficient or improper

physiological responses to environmental factors. However, the lack of understanding of how the immune system is regulated or differentiates has blocked the ability to advantageously modulate the normal defensive mechanisms to biological challenges. Medical conditions characterized by abnormal or inappropriate regulation of the development or physiology of relevant cells thus remain unmanageable. The discovery and characterization of specific cytokines and markers, e.g., involved in cell-cell interactions, will contribute to the development of therapies for a broad range of degenerative or other conditions which affect the immune system, hematopoietic cells, as well as other cell types. The present invention provides solutions to some of these and many other problems.

SUMMARY OF THE INVENTION

The present invention embraces compositions related to proteins which function in controlling physiology, development, and differentiation of mammalian cells, e.g., cells of a mammalian immune system. In particular, it provides proteins and mimetics which regulate cellular physiology, development, differentiation, or function of various cell types, including hematopoietic cells, and particularly natural killer (NK) and T cells.

The present invention is based, in part, upon the discovery of a cDNA clone encoding a member of a family of cell surface markers, initially characterized on natural killer (NK) and T cells and designated DX1. The invention embraces isolated genes encoding the proteins of the invention, variants of the encoded proteins, e.g., mutations (muteins) of the natural sequence, species and allelic variants, fusion proteins, chemical mimetics, antibodies, and other structural or functional analogs. Various uses of

these different nucleic acid or protein compositions are also provided.

The present invention provides nucleic acids encoding a DX1 protein or fragment thereof; a substantially pure DX1 or peptide thereof, or a fusion protein comprising DX1 sequence; and an antibody raised to a DX1 protein.

In nucleic acid embodiments, the nucleic acid can comprise a sequence of SEQ ID NO: 1.

In substantially pure DX1 protein or peptide thereof embodiments, the protein or peptide can be from a primate, including a human; comprise at least one polypeptide segment of SEQ ID NO: 2; or exhibit a post-translational modification pattern distinct from natural DX1 protein. A further embodiment is a composition comprising such a protein and a pharmaceutically acceptable carrier.

In antibody embodiments, the antigen can be a primate protein, including a human; the antibody is raised against a protein sequence of SEQ ID NO: 2; the antibody is a monoclonal antibody; or the antibody is labeled.

The invention also embraces a kit comprising a substantially pure nucleic acid encoding a DX1 protein or peptide; a substantially pure DX1 protein or fragment, e.g., as a positive control; or an antibody or receptor which specifically binds a DX1 protein.

Methods for screening for ligands or other proteins which specifically bind to DX1 are also provided.

The availability of these reagents also provides methods of modulating physiology or development of a cell comprising contacting said cell with an agonist or antagonist of a DX1 protein. For example, the antagonist might be an antibody against a mammalian DX1 protein or the cell may be a hematopoietic cell, including a lymphoid cell.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

All references cited herein are incorporated in their entirety by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

General

The present invention provides DNA sequence encoding various mammalian proteins which exhibit properties characteristic of functionally significant NK and T cell expressed molecules. The cDNA sequence exhibits various features which are characteristic of mRNAs encoding physiologically and developmentally important cell markers. See, e.g., Yokoyama (1993) Ann. Rev. Immunol. 11:613-35. The human gene described herein contains an open reading frame encoding a presumptive 226 amino acid protein. The glycoprotein identified by the DX1 mAb is a human homolog of rodent NKR-P1, as determined by comparison of the predicted polypeptide sequences of the human and rodent molecules. As such, the DX1 protein cloned herein likely represents one member of a class of related genes.

These proteins are designated DX1 proteins. The natural proteins should be capable of mediating various physiological responses which would lead to biological or physiological responses in target cells. Initial studies had localized this protein to various hematopoietic cell types. See, e.g., Table 1. Biochemical properties are described in Table 2.

Table 1: Distribution of DX1 markers.

60-99% of human peripheral blood $CD3^-56^+$ NK cells
< 0.2% fetal and postnatal thymocytes
< 2% cord blood T cells
~20% adult T cells, including CD4 and CD8 $\alpha\beta$-TcR and $\gamma\delta$-TcR T cells; correlated with "memory" subset
not detected on B cells, monocytes, or granulocytes

Table 2: Biochemical Properties of DX1 markers.

	disulfide-linked homodimer 80 kD non-reduced; 40 kD subunits
	complex and high mannose carbohydrates
	core protein ~28 kD
	no serine or tyrosine phosphorylation detected

Nucleic Acids

The nucleotide and amino acid sequences of one protein of the DX-1 family are disclosed in SEQ ID NO: 1 and 2, respectively. The described nucleotide sequences and the related reagents are useful in constructing a DNA clone useful for expressing DX1 protein, or, e.g., isolating a homologous gene from another natural source, including other members of the family. Typically, the sequences will be useful in isolating other genes, e.g., allelic variants or alternatively spliced isoforms, from human.

The purified protein or defined peptides are useful for generating antibodies by standard methods, as described above. Synthetic peptides or purified protein can be presented to an immune system to generate a specific binding composition, e.g., monoclonal or polyclonal antibodies. See, e.g., Coligan (1991) Current Protocols in Immunology Wiley/Greene; and Harlow and Lane (1989) Antibodies: A Laboratory Manual, Cold Spring Harbor Press.

For example, the specific binding composition could be used for screening of an expression library made from a cell line which expresses a DX1 protein. The screening can be standard staining of surface expressed protein, or by panning. Screening of intracellular expression can also be performed by various staining or immunofluorescence procedures. The binding compositions could be used to affinity purify or sort out cells expressing the protein.

This invention contemplates use of isolated DNA or fragments to encode a biologically active DX1 protein or polypeptide. In addition, this invention covers isolated or

recombinant DNA which encodes a biologically active protein or polypeptide and which is capable of hybridizing under appropriate conditions with the DNA sequences described herein. Said biologically active protein or polypeptide can be an intact antigen, or fragment, and have an amino acid sequence as disclosed in SEQ ID NO: 2. Furthermore, this invention covers the use of isolated or recombinant DNA, or fragments thereof, which encode proteins which are homologous to a DX1 protein or which were isolated using cDNA encoding a DX1 protein as a probe. The isolated DNA can have the respective regulatory sequences in the 5' and 3' flanks, e.g., promoters, enhancers, poly-A addition signals, and others.

An "isolated" nucleic acid is a nucleic acid, e.g., an RNA, DNA, or a mixed polymer, which is substantially separated from other components which naturally accompany a native sequence, e.g., ribosomes, polymerases, and flanking genomic sequences from the originating species. The term embraces a nucleic acid sequence which has been removed from its naturally occurring environment, and includes recombinant or cloned DNA isolates and chemically synthesized analogs or analogs biologically synthesized by heterologous systems.

Generally, such a nucleic acid will be less than about 50 kb, typically less than about 30 kb, ordinarily less than about 15 kb, usually less than about 10 kb, in preferred embodiments, less than about 5 kb and more preferably, even smaller. A substantially pure molecule includes isolated forms of the molecule. Alternatively, a purified species may be separated from host components from a recombinant expression system.

An isolated nucleic acid will generally be a homogeneous composition of molecules, but will, in some embodiments, contain minor heterogeneity. This heterogeneity is typically found at the polymer ends or portions not critical to a desired biological function or activity.

A "recombinant" nucleic acid is defined either by its method of production or its structure. In reference to its method of production, e.g., a product made by a process, the

process is use of recombinant nucleic acid techniques, e.g., involving human intervention in the nucleotide sequence, typically selection or production. Alternatively, it can be a nucleic acid made by generating a sequence comprising fusion of two fragments which are not naturally contiguous to each other, but is meant to exclude products of nature, e.g., naturally occurring mutants. Thus, for example, products made by transforming cells with any unnaturally occurring vector is encompassed, as are nucleic acids comprising sequence derived using any synthetic oligonucleotide process. This is often done to replace a codon with a redundant codon encoding the same or a conservative amino acid, while typically introducing or removing a sequence recognition site.

Alternatively, it is performed to join together nucleic acid segments of desired functions to generate a single genetic entity comprising a desired combination of functions not found in the commonly available natural forms. Restriction enzyme recognition sites are often the target of such artificial manipulations, but other site specific targets, e.g., promoters, DNA replication sites, regulation sequences, control sequences, or other useful features may be incorporated by design. A similar concept is intended for a recombinant, e.g., fusion, polypeptide. Specifically included are synthetic nucleic acids which, by genetic code redundancy, encode polypeptides similar to fragments of these antigens, and fusions of sequences from various different species variants.

A significant "fragment" in a nucleic acid context is a contiguous segment of at least about 17 nucleotides, generally at least 23 nucleotides, ordinarily at least 29 nucleotides, often at least 35 nucleotides, typically at least 41 nucleotides, usually at least 47 nucleotides, preferably at least 53 nucleotides, and in particularly preferred embodiments will be at least 60 or more nucleotides.

A DNA which codes for a DX1 protein will be particularly useful to identify genes, mRNA, and cDNA species which code for related or homologous proteins, as well as DNAs which code for

homologous proteins. There are likely homologues in other primates. Various DX1 proteins should be homologous and are encompassed herein. However, even proteins that have a more distant evolutionary relationship to the antigen can readily be isolated under appropriate conditions using these sequences if they are sufficiently homologous. Primate DX1 proteins are of particular interest.

This invention further covers recombinant DNA molecules and fragments having a DNA sequence identical to or highly homologous to the isolated DNAs set forth herein. In particular, the sequences will often be operably linked to DNA segments which control transcription, translation, and DNA replication. Alternatively, recombinant clones derived from the genomic sequences, e.g., containing introns, will be useful for transgenic studies, including, e.g., transgenic cells and organisms, and for gene therapy. See, e.g., Goodnow (1992) "Transgenic Animals" in Roitt (ed.) Encyclopedia of Immunology Academic Press, San Diego, pp. 1502-1504; Travis (1992) Science 256:1392-1394; Kuhn, et al. (1991) Science 254:707-710; Capecchi (1989) Science 244:1288; Robertson (1987)(ed.) Teratocarcinomas and Embryonic Stem Cells: A Practical Approach IRL Press, Oxford; and Rosenberg (1992) J. Clinical Oncology 10:180-199.

Homologous nucleic acid sequences, when compared, exhibit substantial similarity. The standards for homology in nucleic acids are either measures for homology generally used in the art by sequence comparison or based upon hybridization conditions. The hybridization conditions are described in greater detail below.

Substantial homology in the nucleic acid sequence comparison context means either that the segments, or their complementary strands, when compared, are identical when optimally aligned, with appropriate nucleotide insertions or deletions, in at least about 50% of the nucleotides, generally at least 57%, ordinarily at least 65%, often at least 71%, typically at least 77%, usually at least about 85%, preferably

at least about 95 to 98% or more, and in particular embodiments, as high as about 99% or more of the nucleotides.

Alternatively, substantial homology exists when the segments will hybridize under selective hybridization conditions, to a strand, or its complement, typically using a sequence SEQ ID NO: 1. Typically, selective hybridization will occur when there is at least about 55% homology over a stretch of at least about 14 nucleotides, preferably at least about 65%, more preferably at least about 75%, and most preferably at least about 90%. See, Kanehisa (1984) Nuc. Acids Res. 12:203-213.

The length of homology comparison, as described, may be over longer stretches, and in certain embodiments will be over a stretch of at least about 17 nucleotides, usually at least about 24 nucleotides, typically at least about 40 nucleotides, preferably at least about 75 to 100 or more nucleotides. Stringent conditions, in referring to homology in the hybridization context, will be stringent combined conditions of salt, temperature, organic solvents, and other parameters, typically those controlled in hybridization reactions. Stringent temperature conditions will usually include temperatures in excess of about 30° C, typically in excess of about 45° C, preferably in excess of about 65° C, and more preferably in excess of about 70° C. Stringent salt conditions will ordinarily be less than about 1000 mM, usually less than about 500 mM, typically less than about 300 mM, and preferably less than about 150 mM. However, the combination of parameters is much more important than the measure of any single parameter. See, e.g., Wetmur and Davidson (1968) J. Mol. Biol. 31:349-370.

Purified DX1 protein

The predicted amino acid sequence of one human DX1 is shown in SEQ ID NO: 2. The peptide sequences allow preparation of peptides to generate antibodies to recognize such segments. As used herein, DX1 shall encompass, when used in a protein context, a protein comprising amino acid sequences shown in SEQ ID NO: 2, or a significant fragment of such a protein. It also

refers to a primate, e.g., human, derived polypeptide which exhibits similar biological function or interacts with DX1 protein specific binding components. These binding components, e.g., antibodies, typically bind to a DX1 protein with high affinity, e.g., at least about 100 nM, usually better than about 30 nM, preferably better than about 10 nM, and more preferably at better than about 3 nM.

The term polypeptide, as used herein, includes a significant fragment or segment, and encompasses a stretch of amino acid residues of at least about 8 amino acids, generally at least 12 amino acids, often 16 amino acids, typically at least 20 amino acids, usually 24 amino acids, preferably at least 28 amino acids, and, in particularly preferred embodiments, at least about 30 or more amino acids.

Substantially pure, in the polypeptide context, typically means that the protein is free from other contaminating proteins, nucleic acids, and other biologicals derived from the original source organism. Purity may be assayed by standard methods, and will ordinarily be at least about 40% pure, generally about 70% pure, often at least about 80% pure, typically at least about 90% pure, preferably at least about 98% pure, and in most preferred embodiments, at least 99% pure. The analysis may be weight or molar percentages, evaluated, e.g., by gel staining, spectrophotometry, or terminus labeling.

A binding composition refers to molecules that bind with specificity to DX1 protein, e.g., in a ligand-receptor type fashion, an antibody-antigen interaction, or compounds, e.g., proteins which specifically associate with DX1 protein, e.g., in a natural physiologically relevant protein-protein interaction, either covalent or non-covalent. The molecule may be a polymer, or chemical reagent. No implication as to whether DX1 protein is either the ligand or the receptor of a ligand-receptor interaction is represented, other than the interaction exhibit similar specificity, e.g., specific affinity. A functional analog may be a protein with structural modifications, or may be a wholly unrelated molecule, e.g., which has a molecular shape

which interacts with the appropriate binding determinants. The proteins may serve as agonists or antagonists of a receptor, see, e.g., Goodman, et al. (eds.) (1990) Goodman & Gilman's: The Pharmacological Bases of Therapeutics (8th ed.), Pergamon Press.

Solubility of a polypeptide or fragment depends upon the environment and the polypeptide. Many parameters affect polypeptide solubility, including temperature, electrolyte environment, size and molecular characteristics of the polypeptide, and nature of the solvent. Typically, the temperature at which the polypeptide is used ranges from about 4° C to about 65° C. Usually the temperature at use is greater than about 18° C and more usually greater than about 22° C. For diagnostic purposes, the temperature will usually be about room temperature or warmer, but less than the denaturation temperature of components in the assay. For therapeutic purposes, the temperature will usually be body temperature, typically about 37° C for humans, though under certain situations the temperature may be raised or lowered in situ or in vitro.

The electrolytes will usually approximate in situ physiological conditions, but may be modified to higher or lower ionic strength where advantageous. The actual ions may be modified, e.g., to conform to standard buffers used in physiological or analytical contexts.

The size and structure of the polypeptide should generally be in a substantially stable state, and usually not in a denatured state. The polypeptide may be associated with other polypeptides in a quaternary structure, e.g., to confer solubility, or associated with lipids or detergents in a manner which approximates natural lipid bilayer interactions.

The solvent will usually be a biologically compatible buffer, of a type used for preservation of biological activities, and will usually approximate a physiological solvent. Usually the solvent will have a neutral pH, typically between about 5 and 10, and preferably about 7.5. On some

occasions, a detergent will be added, typically a mild non-denaturing one, e.g., CHS or CHAPS, or a low enough concentration as to avoid significant disruption of structural or physiological properties of the antigen.

Making DX1 protein: Mimetics

DNA which encodes the DX1 protein or fragments thereof can be obtained by chemical synthesis, screening cDNA libraries, or by screening genomic libraries prepared from a wide variety of cell lines or tissue samples. See, e.g., Okayama and Berg (1982) Mol. Cell. Biol. 2:161-170; Gubler and Hoffman (1983) Gene 25:263-269; and Glover (ed.) (1984) DNA Cloning: A Practical Approach, IRL Press, Oxford.

This DNA can be expressed in a wide variety of host cells for the synthesis of a full-length protein or fragments which can in turn, for example, be used to generate polyclonal or monoclonal antibodies; for binding studies; for construction and expression of modified molecules; and for structure/function studies.

Vectors, as used herein, comprise plasmids, viruses, bacteriophage, integratable DNA fragments, and other vehicles which enable the integration of DNA fragments into the genome of the host. Expression vectors are specialized vectors which contain genetic control elements that effect expression of operably linked genes. Plasmids are the most commonly used form of vector but all other forms of vectors which serve an equivalent function and which are, or become, known in the art are suitable for use herein. See, e.g., Pouwels, et al. (1985 and Supplements) Cloning Vectors: A Laboratory Manual, Elsevier, N.Y., and Rodriquez, et al. (1988)(eds.) Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Buttersworth, Boston, MA.

For purposes of this invention, DNA sequences are operably linked when they are functionally related to each other. For example, DNA for a presequence or secretory leader is operably linked to a polypeptide if it is expressed as a preprotein or

participates in directing the polypeptide to the cell membrane or in secretion of the polypeptide. A promoter is operably linked to a coding sequence if it controls the transcription of the polypeptide; a ribosome binding site is operably linked to a coding sequence if it is positioned to permit translation. Usually, operably linked means contiguous and in reading frame, however, certain genetic elements such as repressor genes are not contiguously linked but still bind to operator sequences that in turn control expression. See, e.g., Balbas and Bolivar (1990) Methods in Enzymology, 185:14-37; and Ausubel, et al. (1993) Current Protocol in Molecular Biology Greene/Wiley, NY.

Representative examples of suitable expression vectors include pCDNA1; pCD, see Okayama, et al. (1985) Mol. Cell Biol. 5:1136-1142; pMC1neo Poly-A, see Thomas, et al. (1987) Cell 51:503-512; and a baculovirus vector such as pAC 373 or pAC 610. See, e.g., Miller (1988) Ann. Rev. Microbiol. 42:177-199.

It will often be desired to express a DX1 protein polypeptide in a system which provides a specific or defined glycosylation pattern. See, e.g., Luckow and Summers (1988) Bio/Technology 6:47-55; and Kaufman (1990) Meth. Enzymol. 185:487-511.

The DX1 protein, or a fragment thereof, may be engineered to be phosphatidyl inositol (PI) linked to a cell membrane, but can be removed from membranes by treatment with a phosphatidyl inositol cleaving enzyme, e.g., phosphatidyl inositol phospholipase-C. This releases the antigen in a biologically active form, and allows purification by standard procedures of protein chemistry. See, e.g., Low (1989) Biochim. Biophys. Acta 988:427-454; Tse, et al. (1985) Science 230:1003-1008; and Brunner, et al. (1991) J. Cell Biol. 114:1275-1283.

Now that the DX1 protein has been characterized, fragments or derivatives thereof can be prepared by conventional processes for synthesizing peptides. These include processes such as are described in Stewart and Young (1984) Solid Phase Peptide Synthesis, Pierce Chemical Co., Rockford, IL; Bodanszky and Bodanszky (1984) The Practice of Peptide Synthesis, Springer-

Verlag, New York; and Bodanszky (1984) The Principles of Peptide Synthesis, Springer-Verlag, New York; and Villafranca (ed.) (1991) Techniques in Protein Chemistry II Academic Press, San Diego, CA.

Physical Variants

This invention also encompasses proteins or peptides having substantial amino acid sequence homology with the amino acid sequence of the DX1 protein. The variants include species and allelic variants.

Amino acid sequence homology, or sequence identity, is determined by optimizing residue matches, if necessary, by introducing gaps as required. This changes when considering conservative substitutions as matches. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. Homologous amino acid sequences are typically intended to include natural allelic and interspecies variations in each respective protein sequence.

Typical homologous proteins or peptides will have from 25-100% homology (if gaps can be introduced), to 50-100% homology (if conservative substitutions are included) with the amino acid sequence of the DX1 protein. Homology measures will be at least about 35%, generally 45%, often at least 55%, typically at least 65%, usually at least 75%, preferably at least 80%, and in particularly preferred embodiments, at least 85% or more. See also Needleham, et al. (1970) J. Mol. Biol. 48:443-453; Sankoff, et al. (1983) Chapter One in Time Warps, String Edits, and Macromolecules: The Theory and Practice of Sequence Comparison Addison-Wesley, Reading, MA; and software packages from IntelliGenetics, Mountain View, CA; and the University of Wisconsin Genetics Computer Group, Madison, WI.

The isolated DNA encoding a DX1 protein can be readily modified by nucleotide substitutions, nucleotide deletions,

nucleotide insertions, and inversions of nucleotide stretches. These modifications result in novel DNA sequences which encode these antigens, their derivatives, or proteins having similar physiological, immunogenic, or antigenic activity. These modified sequences can be used to produce mutant antigens or to enhance expression. Enhanced expression may involve gene amplification, increased transcription, increased translation, and other mechanisms. Such mutant DX1 protein derivatives include predetermined or site-specific mutations of the respective protein or its fragments.

"Mutant DX1 protein" encompasses a polypeptide otherwise falling within the homology definition of the human DX1 protein as set forth above, but having an amino acid sequence which differs from that of DX1 protein as found in nature, whether by way of deletion, substitution, or insertion. In particular, "site specific mutant DX1 protein" generally includes proteins having significant homology with a protein having sequences of SEQ ID NO: 2, and as sharing various biological activities, e.g., antigenic or immunogenic, with those sequences, and in preferred embodiments contain most of the disclosed sequences. Similar concepts apply to different DX1 proteins, particularly those found in various mammals, e.g., primates, including human. As stated before, it is emphasized that descriptions are generally meant to encompass all DX1 proteins, not limited to the specific embodiment discussed.

DX1 protein mutagenesis can be conducted by making amino acid insertions or deletions. Substitutions, deletions, insertions, or any combinations may be generated to arrive at a final construct. Insertions include amino- or carboxy- terminal fusions. Random mutagenesis can be conducted at a target codon and the expressed mutants can then be screened for the desired activity. Methods for making substitution mutations at predetermined sites in DNA having a known sequence are well known in the art, e.g., by M13 primer mutagenesis or polymerase chain reaction (PCR) techniques. See also Sambrook, et al.

(1989); Ausubel, et al. (1987 and Supplements); and Kunkel, et al. (1987) Meth. Enzymol. 154:367-382.

The mutations in the DNA normally should not place coding sequences out of reading frames and preferably will not create complementary regions that could hybridize to produce secondary mRNA structure such as loops or hairpins.

The present invention also provides recombinant proteins, e.g., heterologous fusion proteins using segments from these proteins. A heterologous fusion protein is a fusion of proteins or segments which are naturally not normally fused in the same manner. Thus, the fusion product of an immunoglobulin with a DX1 polypeptide is a continuous protein molecule having sequences fused in a typical peptide linkage, typically made as a single translation product and exhibiting properties derived from each source peptide. A similar concept applies to heterologous nucleic acid sequences.

In addition, new constructs may be made from combining similar functional domains from other proteins. For example, antigen-binding or other segments may be "swapped" between different new fusion polypeptides or fragments. See, e.g., Cunningham, et al. (1989) Science 243:1330-1336; and O'Dowd, et al. (1988) J. Biol. Chem. 263:15985-15992. Thus, new chimeric polypeptides exhibiting new combinations of specificities will result from the functional linkage of biologically relevant domains and other functional domains.

The phosphoramidite method described by Beaucage and Carruthers (1981) Tetra. Letts. 22:1859-1862, will produce suitable synthetic DNA fragments. A double stranded fragment will often be obtained either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence, e.g., PCR techniques.

Functional Variants

The blocking of physiological response to DX1 proteins may result from the inhibition of binding of the antigen to its natural binding partner, e.g., through competitive inhibition. Thus, in vitro assays of the present invention will often use isolated protein, membranes from cells expressing a recombinant membrane associated DX1 protein, soluble fragments comprising binding segments, or fragments attached to solid phase substrates. These assays will also allow for the diagnostic determination of the effects of either binding segment mutations and modifications, or protein mutations and modifications, e.g., analogs. In particular, the DX1 is stably expressed on NK clones, but the antigen is lost after T cell activation (T cell clones are negative).

This invention also contemplates the use of competitive drug screening assays, e.g., where neutralizing antibodies to antigen or binding partner fragments compete with a test compound for binding to the protein.

Additionally, neutralizing antibodies against the DX1 protein and soluble fragments of the antigen which contain a high affinity receptor binding site, can be used to inhibit antigen function in tissues, e.g., tissues experiencing abnormal physiology.

"Derivatives" of the DX1 antigens include amino acid sequence mutants, glycosylation variants, and covalent or aggregate conjugates with other chemical moieties. Covalent derivatives can be prepared by linkage of functionalities to groups which are found in the DX1 amino acid side chains or at the N- or C- termini, by means which are well known in the art. See, e.g., Lundblad and Noyes (1988) Chemical Reagents for Protein Modification, CRC Press, Inc., Boca Raton, FL, Vol I and II; Hugli (ed.) (1989) Techniques in Protein Chemistry, Academic Press, San Diego, CA; and Wong (1991) Chemistry of Protein Conjugation and Cross Linking, CRC Press, Boca Raton, FL.

In particular, glycosylation alterations are included, e.g., made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing, or in further

processing steps. See, e.g., Elbein (1987) Ann. Rev. Biochem. 56:497-534. Also embraced are versions of the same primary amino acid sequence which have other minor modifications, including phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine.

Fusion polypeptides between the DX1 proteins and other homologous or heterologous proteins are also provided. Homologous polypeptides may be fusions between different surface markers, resulting in, e.g., a hybrid protein exhibiting receptor binding specificity. Likewise, heterologous fusions may be constructed which would exhibit a combination of properties or activities of the derivative proteins. Typical examples are fusions of a reporter polypeptide, e.g., luciferase, with a segment or domain of aa antigen, e.g., a receptor-binding segment, so that the presence or location of the fused antigen may be easily determined. See, e.g., Dull, et al., U.S. Patent No. 4,859,609. Other gene fusion partners include bacterial ß-galactosidase, trpE, Protein A, ß-lactamase, alpha amylase, alcohol dehydrogenase, and yeast alpha mating factor. See, e.g., Godowski, et al. (1988) Science 241:812-816.

Fusion proteins will typically be made by either recombinant nucleic acid methods or by synthetic polypeptide methods. Techniques for nucleic acid manipulation and expression are described generally, e.g., in Sambrook, et al. (1989) Molecular Cloning: A Laboratory Manual (2d ed.), Vols. 1-3, Cold Spring Harbor Laboratory. Techniques for synthesis of polypeptides are described, e.g., in Merrifield (1963) J. Amer. Chem. Soc. 85:2149-2156; Merrifield (1986) Science 232:341-347; Atherton, et al. (1989) Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, Oxford; and Grant (1992) Synthetic Peptides: A User's Guide, W.H. Freeman, NY.

This invention also contemplates the use of derivatives of the DX1 proteins other than variations in amino acid sequence or glycosylation. Such derivatives may involve covalent or aggregative association with chemical moieties. These derivatives generally fall into the three classes: (1) salts,

(2) side chain and terminal residue covalent modifications, and (3) adsorption complexes, for example with cell membranes. Such covalent or aggregative derivatives are useful as immunogens, as reagents in immunoassays, or in purification methods such as for affinity purification of antigens or other binding proteins. For example, a DX1 antigen can be immobilized by covalent bonding to a solid support such as cyanogen bromide-activated SEPHAROSE, by methods which are well known in the art, or adsorbed onto polyolefin surfaces, with or without glutaraldehyde cross-linking, for use in the assay or purification of anti-DX1 protein antibodies or its receptor or other binding partner.

The DX1 antigens can also be labeled with a detectable group, for example radioiodinated by the chloramine T procedure, covalently bound to rare earth chelates, or conjugated to another fluorescent moiety for use in diagnostic assays. Purification of DX1 protein may be effected by immobilized antibodies or binding partners.

A solubilized DX1 antigen or fragment of this invention can be used as an immunogen for the production of antisera or antibodies specific for the protein or fragments thereof. The purified antigen can be used to screen monoclonal antibodies or binding fragments prepared by immunization with various forms of impure preparations containing the protein. In particular, the term "antibodies" also encompasses antigen binding fragments of natural antibodies.

The purified DX1 proteins can also be used as a reagent to detect any antibodies generated in response to the presence of elevated levels of the protein or cell fragments containing the antigen, both of which may be diagnostic of an abnormal or specific physiological or disease condition. Additionally, antigen fragments may also serve as immunogens to produce the antibodies of the present invention, as described immediately below. For example, this invention contemplates antibodies raised against amino acid sequences encoded by nucleotide sequences shown in SEQ ID NO: 1, or fragments of proteins

containing them. In particular, this invention contemplates antibodies having binding affinity to or being raised against specific fragments which are predicted to lie outside of the lipid bilayer.

The invention also provides means to isolate a group of related antigens displaying both distinctness and similarities in structure, expression, and function. Elucidation of many of the physiological effects of the antigens will be greatly accelerated by the isolation and characterization of distinct species variants. In particular, the present invention provides useful probes for identifying additional homologous genetic entities in different species.

The isolated genes will allow transformation of cells lacking expression of a corresponding DX1 protein, e.g., either species types or cells which lack corresponding antigens and should exhibit negative background activity. Expression of transformed genes will allow isolation of antigenically pure cell lines, with defined or single specie variants. This approach will allow for more sensitive detection and discrimination of the physiological effects of DX1 proteins. Subcellular fragments, e.g., cytoplasts or membrane fragments, can be isolated and used.

Dissection of the critical structural elements which effect the various physiological or differentiation functions provided by the proteins is possible using standard techniques of modern molecular biology, particularly in comparing members of the related class. See, e.g., the homolog-scanning mutagenesis technique described in Cunningham, et al. (1989) Science 243:1339-1336; and approaches used in O'Dowd, et al. (1988) J. Biol. Chem. 263:15985-15992; and Lechleiter, et al. (1990) EMBO J. 9:4381-4390.

In particular, functional domains or segments can be substituted between species variants or related proteins to determine what structural features are important in both binding partner affinity and specificity, as well as signal transduction. An array of different variants will be used to

screen for molecules exhibiting combined properties of interaction with different species variants of binding partners.

Antigen internalization may occur under certain circumstances, and interaction between intracellular components and "extracellular" segments of proteins involved in interactions may occur. The specific segments of interaction of DX1 protein with other intracellular components may be identified by mutagenesis or direct biochemical means, e.g., cross-linking or affinity methods. Structural analysis by crystallographic or other physical methods will also be applicable. Further investigation of the mechanism of biological function will include study of associated components which may be isolatable by affinity methods or by genetic means, e.g., complementation analysis of mutants.

Further study of the expression and control of DX1 protein will be pursued. The controlling elements associated with the antigens may exhibit differential developmental, tissue specific, or other expression patterns. Upstream or downstream genetic regions, e.g., control elements, are of interest.

Structural studies of the antigen will lead to design of new variants, particularly analogs exhibiting agonist or antagonist properties on binding partners. This can be combined with previously described screening methods to isolate variants exhibiting desired spectra of activities.

Expression in other cell types will often result in glycosylation differences in a particular antigen. Various species variants may exhibit distinct functions based upon structural differences other than amino acid sequence. Differential modifications may be responsible for differential function, and elucidation of the effects are now made possible.

Thus, the present invention provides important reagents related to antigen-binding partner interaction. Although the foregoing description has focused primarily upon the human DX1 protein, those of skill in the art will immediately recognize that the invention encompasses other closely related antigens,

e.g., other primate species or allelic variants, as well as variants and other members of the family.

Antibodies

Antibodies can be raised to the various DX1 proteins, including species or allelic variants, and fragments thereof, both in their naturally occurring forms and in their recombinant forms. Additionally, antibodies can be raised to DX1 proteins in either their active forms or in their inactive forms. Anti-idiotypic antibodies are also contemplated.

Antibodies, including binding fragments and single chain versions, against predetermined fragments of the antigens can be raised by immunization of animals with conjugates of the fragments with immunogenic proteins. Monoclonal antibodies are prepared from cells secreting the desired antibody. These antibodies can be screened for binding to normal or defective DX1 proteins, or screened for agonistic or antagonistic activity, e.g., mediated through a binding partner. These monoclonal antibodies will usually bind with at least a K_D of about 1 mM, typically at least about 30 µM, preferably at least about 3 µM or better.

The antibodies, including antigen binding fragments, of this invention can have significant diagnostic or therapeutic value. They can be potent antagonists that bind to a binding partner and inhibit antigen binding or inhibit the ability of an antigen to elicit a biological response. They also can be useful as non-neutralizing antibodies and can be coupled to toxins or radionuclides so that when the antibody binds to the antigen, a cell expressing it, e.g., on its surface, is killed. Further, these antibodies can be conjugated to drugs or other therapeutic agents, either directly or indirectly by means of a linker, and may effect drug targeting.

The antibodies of this invention can also be useful in diagnostic applications. As capture or non-neutralizing antibodies, they can be screened for ability to bind to the antigens without inhibiting binding by a partner. As

neutralizing antibodies, they can be useful in competitive binding assays. They will also be useful in detecting or quantifying DX1 protein or its binding partners. See e.g., Chan (ed.) (1987) Immunology: A Practical Guide, Academic Press, Orlando, FLA; Price and Newman (eds.) (1991) Principles and Practice of Immunoassay, Stockton Press, N.Y.; and Ngo (ed.) (1988) Nonisotopic Immunoassay, Plenum Press, N.Y.

Antigen fragments may be joined to other materials, particularly polypeptides, as fused or covalently joined polypeptides to be used as immunogens. An antigen and its fragments may be fused or covalently linked to a variety of immunogens, such as keyhole limpet hemocyanin, bovine serum albumin, tetanus toxoid, etc. See Microbiology, Hoeber Medical Division, Harper and Row, 1969; Landsteiner (1962) Specificity of Serological Reactions, Dover Publications, New York, and Williams, et al. (1967) Methods in Immunology and Immunochemistry, Vol. 1, Academic Press, New York, for descriptions of methods of preparing polyclonal antisera.

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans, etc. Description of techniques for preparing such monoclonal antibodies may be found in, e.g., Stites, et al. (eds.) Basic and Clinical Immunology (4th ed.), Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane (1988) Antibodies: A Laboratory Manual, CSH Press; Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed.) Academic Press, New York; and particularly in Kohler and Milstein (1975) in Nature 256: 495-497, which discusses one method of generating monoclonal antibodies.

Other suitable techniques involve in vitro exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors. See, Huse, et al. (1989) "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda," Science 246:1275-1281; and Ward, et al. (1989) Nature 341:544-546. The polypeptides and antibodies of the present

invention may be used with or without modification, including chimeric or humanized antibodies.

Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents, teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced, see Cabilly, U.S. Patent No. 4,816,567.

The antibodies of this invention can also be used for affinity chromatography in isolating the protein. Columns can be prepared where the antibodies are linked to a solid support. See, e.g., Wilchek, et al., (1984) Meth. Enzymol. 104:3-55.

The antibodies may also be used to screen expression libraries for particular expression products. Usually the antibodies used in such a procedure will be labeled with a moiety allowing easy detection of presence of antigen by antibody binding.

Antibodies raised against each DX1 protein will also be useful to raise anti-idiotypic antibodies. These will be useful in detecting or diagnosing various immunological conditions related to expression of the respective antigens.

Uses

The present invention provides reagents which will find use in diagnostic applications as described elsewhere herein, e.g., in the general description for physiological or developmental abnormalities, or below in the description of kits for diagnosis.

This invention also provides reagents with significant therapeutic value. The DX1 protein (naturally occurring or

recombinant), fragments thereof, and antibodies thereto, along with compounds identified as having binding affinity to DX1 protein, should be useful in the treatment of conditions associated with abnormal physiology or development, including abnormal proliferation, e.g., cancerous conditions, or degenerative conditions. Abnormal proliferation, regeneration, degeneration, and atrophy may be modulated by appropriate therapeutic treatment using the compositions provided herein. For example, a disease or disorder associated with abnormal expression or abnormal signaling by a DX1 antigen should be a likely target for an agonist or antagonist of the protein.

Other abnormal developmental conditions are known in the cell types shown to possess DX1 antigen mRNA by Northern blot analysis. See Berkow (ed.) The Merck Manual of Diagnosis and Therapy, Merck & Co., Rahway, N.J.; and Thorn, et al. Harrison's Principles of Internal Medicine, McGraw-Hill, N.Y. These problems may be susceptible to prevention or treatment using compositions provided herein. Moreover, as a marker, the DX1 proteins may well be diagnostic of the state of health of a population of NK or T cells.

Recombinant antibodies which bind to DX1 can be purified and then administered to a patient. These reagents can be combined for therapeutic use with additional active or inert ingredients, e.g., in conventional pharmaceutically acceptable carriers or diluents, e.g., immunogenic adjuvants, along with physiologically innocuous stabilizers and excipients. These combinations can be sterile filtered and placed into dosage forms as by lyophilization in dosage vials or storage in stabilized aqueous preparations. This invention also contemplates use of antibodies or binding fragments thereof, including forms which are not complement binding.

Screening using DX1 for binding partners or compounds having binding affinity to DX1 antigen can be performed, including isolation of associated components. Subsequent biological assays can then be utilized to determine if the compound has intrinsic biological activity and is therefore an

agonist or antagonist in that it blocks an activity of the antigen. This invention further contemplates the therapeutic use of antibodies to DX1 protein as antagonists. This approach should be particularly useful with other DX1 protein species variants and other members of the family.

The quantities of reagents necessary for effective therapy will depend upon many different factors, including means of administration, target site, physiological state of the patient, and other medicants administered. Thus, treatment dosages should be titrated to optimize safety and efficacy. Typically, dosages used in vitro may provide useful guidance in the amounts useful for in situ administration of these reagents.

Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage. Various considerations are described, e.g., in Gilman, et al. (eds.) (1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; and Remington's Pharmaceutical Sciences, 17th ed. (1990), Mack Publishing Co., Easton, Penn. Methods for administration are discussed therein and below, e.g., for oral, intravenous, intraperitoneal, or intramuscular administration, transdermal diffusion, and others. Pharmaceutically acceptable carriers will include water, saline, buffers, and other compounds described, e.g., in the Merck Index, Merck & Co., Rahway, New Jersey.

Dosage ranges would ordinarily be expected to be in amounts lower than 1 mM concentrations, typically less than about 10 µM concentrations, usually less than about 100 nM, preferably less than about 10 pM (picomolar), and most preferably less than about 1 fM (femtomolar), with an appropriate carrier. Slow release formulations, or a slow release apparatus will often be utilized for continuous administration.

DX1 protein, fragments thereof, and antibodies to it or its fragments, antagonists, and agonists, may be administered directly to the host to be treated or, depending on the size of the compounds, it may be desirable to conjugate them to carrier

proteins such as ovalbumin or serum albumin prior to their administration. Therapeutic formulations may be administered in any conventional dosage formulation. While it is possible for the active ingredient to be administered alone, it is preferable to present it as a pharmaceutical formulation.

Formulations typically comprise at least one active ingredient, as defined above, together with one or more acceptable carriers thereof. Each carrier should be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the patient. Formulations include those suitable for oral, rectal, nasal, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. See, e.g., Gilman, et al. (eds.) (1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; Remington's Pharmaceutical Sciences, 17th ed. (1990), Mack Publishing Co., Easton, Penn; Avis, et al. (eds.) (1993) Pharmaceutical Dosage Forms: Parenteral Medications, Dekker, NY; Lieberman, et al. (eds.) (1990) Pharmaceutical Dosage Forms: Tablets, Dekker, NY; and Lieberman, et al. (eds) (1990) Pharmaceutical Dosage Forms: Disperse Systems, Dekker, NY. The therapy of this invention may be combined with or used in association with other chemotherapeutic or chemopreventive agents.

Both the naturally occurring and the recombinant form of the DX1 proteins of this invention are particularly useful in kits and assay methods which are capable of screening compounds for binding activity to the proteins. Several methods of automating assays have been developed in recent years so as to permit screening of tens of thousands of compounds in a short period. See, e.g., Fodor, et al. (1991) Science 251:767-773, which describes means for testing of binding affinity by a plurality of defined polymers synthesized on a solid substrate and other methodologies which generate structural diversity

libraries. The development of suitable assays can be greatly facilitated by the availability of large amounts of purified, soluble DX1 protein as provided by this invention.

This invention is particularly useful for screening compounds by using recombinant antigen in any of a variety of drug screening techniques. The advantages of using a recombinant protein in screening for specific ligands include: (a) improved renewable source of the antigen from a specific source; (b) potentially greater number of antigen molecules per cell giving better signal to noise ratio in assays; and (c) species variant specificity (theoretically giving greater biological and disease specificity). The purified protein may be tested in numerous assays, typically in vitro assays, which evaluate biologically relevant responses. See, e.g., Coligan Current Protocols in Immunology; Hood, et al. Immunology Benjamin/Cummings; Paul (ed.) Fundamental Immunology; and Methods in Enzymology Academic Press. This will also be useful in screening for a ligand which binds a DX1, e.g., from an interacting cell.

One method of drug screening utilizes eukaryotic or prokaryotic host cells which are stably transformed with recombinant DNA molecules expressing the DX1 antigens. Cells may be isolated which express an antigen in isolation from other functionally equivalent antigens. Such cells, either in viable or fixed form, can be used for standard protein-protein binding assays. See also, Parce, et al. (1989) Science 246:243-247; and Owicki, et al. (1990) Proc. Nat'l Acad. Sci. USA 87:4007-4011, which describe sensitive methods to detect cellular responses.

Competitive assays are particularly useful, where the cells (source of DX1 protein) are contacted and incubated with a labeled binding partner or antibody having known binding affinity to the ligand, such as ^{125}I-antibody, and a test sample whose binding affinity to the binding composition is being measured. The bound and free labeled binding compositions are then separated to assess the degree of antigen binding. The amount of test compound bound is inversely proportional to the

amount of labeled receptor binding to the known source. Any one of numerous techniques can be used to separate bound from free antigen to assess the degree of binding. This separation step could typically involve a procedure such as adhesion to filters followed by washing, adhesion to plastic followed by washing, or centrifugation of the cell membranes.

Viable cells could also be used to screen for the effects of drugs on DX1 protein mediated functions, e.g., second messenger levels, i.e., Ca^{++}; cell proliferation; inositol phosphate pool changes; and others. Some detection methods allow for elimination of a separation step, e.g., a proximity sensitive detection system. Calcium sensitive dyes will be useful for detecting Ca^{++} levels, with a fluorimeter or a fluorescence cell sorting apparatus.

Another method utilizes membranes from transformed eukaryotic or prokaryotic host cells as the source of the DX1 protein. These cells are stably transformed with DNA vectors directing the expression of a membrane associated DX1 protein, e.g., an engineered membrane bound form. Essentially, the membranes would be prepared from the cells and used in any receptor/ligand type binding assay such as the competitive assay set forth above.

Still another approach is to use solubilized, unpurified or solubilized, purified DX1 protein from transformed eukaryotic or prokaryotic host cells. This allows for a "molecular" binding assay with the advantages of increased specificity, the ability to automate, and high drug test throughput.

Another technique for drug screening involves an approach which provides high throughput screening for compounds having suitable binding affinity to DX1 and is described in detail in Geysen, European Patent Application 84/03564, published on September 13, 1984. First, large numbers of different small peptide test compounds are synthesized on a solid substrate, e.g., plastic pins or some other appropriate surface, see Fodor, et al. (1991). Then all the pins are reacted with solubilized, unpurified or solubilized, purified DX1 binding composition, and

washed. The next step involves detecting bound binding composition.

Rational drug design may also be based upon structural studies of the molecular shapes of the DX1 protein and other effectors or analogs. Effectors may be other proteins which mediate other functions in response to antigen binding, or other proteins which normally interact with the antigen, e.g., DX1 ligand. One means for determining which sites interact with specific other proteins is a physical structure determination, e.g., x-ray crystallography or 2 dimensional NMR techniques. These will provide guidance as to which amino acid residues form molecular contact regions. For a detailed description of protein structural determination, see, e.g., Blundell and Johnson (1976) Protein Crystallography, Academic Press, New York.

Purified DX1 protein can be coated directly onto plates for use in the aforementioned drug screening techniques. However, non-neutralizing antibodies to these ligands can be used as capture antibodies to immobilize the respective ligand on the solid phase.

Kits

This invention also contemplates use of DX1 proteins, fragments thereof, peptides, and their fusion products in a variety of diagnostic kits and methods for detecting the presence of a binding composition. Typically the kit will have a compartment containing either a defined DX1 peptide or gene segment or a reagent which recognizes one or the other, e.g., antigen fragments or antibodies.

A kit for determining the binding affinity of a test compound to a DX1 protein would typically comprise a test compound; a labeled compound, for example an antibody having known binding affinity for the antigen; a source of DX1 protein (naturally occurring or recombinant); and a means for separating bound from free labeled compound, such as a solid phase for immobilizing the antigen. Once compounds are screened, those

having suitable binding affinity to the antigen can be evaluated in suitable biological assays, as are well known in the art, to determine whether they exhibit similar biological activities to the natural antigen. The availability of recombinant DX1 protein polypeptides also provide well defined standards for calibrating such assays.

A preferred kit for determining the concentration of, for example, a DX1 protein in a sample would typically comprise a labeled compound, e.g., antibody, having known binding affinity for the antigen, a source of antigen (naturally occurring or recombinant) and a means for separating the bound from free labeled compound, for example, a solid phase for immobilizing the DX1 protein. Compartments containing reagents, and instructions, will normally be provided.

One method for determining the concentration of DX1 protein in a sample would typically comprise the steps of: (1) preparing membranes from a sample comprised of a membrane bound DX1 protein source; (2) washing the membranes and suspending them in a buffer; (3) solubilizing the antigen by incubating the membranes in a culture medium to which a suitable detergent has been added; (4) adjusting the detergent concentration of the solubilized antigen; (5) contacting and incubating said dilution with radiolabeled antibody to form complexes; (6) recovering the complexes such as by filtration through polyethyleneimine treated filters; and (7) measuring the radioactivity of the recovered complexes.

Antibodies, including antigen binding fragments, specific for the DX1 protein or fragments are useful in diagnostic applications to detect the presence of elevated levels of DX1 protein and/or its fragments. Such diagnostic assays can employ lysates, live cells, fixed cells, immunofluorescence, cell cultures, body fluids, and further can involve the detection of antigens related to the protein in serum, or the like. Diagnostic assays may be homogeneous (without a separation step between free reagent and protein-protein complex) or heterogeneous (with a separation step). Various commercial

assays exist, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay (EIA), enzyme-multiplied immunoassay technique (EMIT), substrate-labeled fluorescent immunoassay (SLFIA), and the like. For example, unlabeled antibodies can be employed by using a second antibody which is labeled and which recognizes the antibody to a DX1 protein or to a particular fragment thereof. Similar assays have also been extensively discussed in the literature. See, e.g., Harlow and Lane (1988) Antibodies: A Laboratory Manual, CSH.

Anti-idiotypic antibodies may have similar use to diagnose presence of antibodies against a DX1 protein, as such may be diagnostic of various abnormal states. For example, overproduction of DX1 protein may result in production of various immunological reactions which may be diagnostic of abnormal physiological states, particularly in proliferative cell conditions such as cancer or abnormal differentiation.

Frequently, the reagents for diagnostic assays are supplied in kits, so as to optimize the sensitivity of the assay. For the subject invention, depending upon the nature of the assay, the protocol, and the label, either labeled or unlabeled antibody, or labeled DX1 protein is provided. This is usually in conjunction with other additives, such as buffers, stabilizers, materials necessary for signal production such as substrates for enzymes, and the like. Preferably, the kit will also contain instructions for proper use and disposal of the contents after use. Typically the kit has compartments for each useful reagent. Desirably, the reagents are provided as a dry lyophilized powder, where the reagents may be reconstituted in an aqueous medium providing appropriate concentrations of reagents for performing the assay.

Any of the aforementioned constituents of the drug screening and the diagnostic assays may be used without modification or may be modified in a variety of ways. For example, labeling may be achieved by covalently or non-covalently joining a moiety which directly or indirectly

provides a detectable signal. In any of these assays, the antigen, test compound, DX1 protein, or antibodies thereto can be labeled either directly or indirectly. Possibilities for direct labeling include label groups: radiolabels such as ^{125}I, enzymes (U.S. Pat. No. 3,645,090) such as peroxidase and alkaline phosphatase, and fluorescent labels (U.S. Pat. No. 3,940,475) capable of monitoring the change in fluorescence intensity, wavelength shift, or fluorescence polarization.

Possibilities for indirect labeling include biotinylation of one constituent followed by binding to avidin coupled to one of the above label groups.

There are also numerous methods of separating the bound from the free antigen, or alternatively the bound from the free test compound. The DX1 protein can be immobilized on various matrixes followed by washing. Suitable matrixes include plastic such as an ELISA plate, filters, and beads. Methods of immobilizing the DX1 protein to a matrix include, without limitation, direct adhesion to plastic, use of a capture antibody, chemical coupling, and biotin-avidin. The last step in this approach involves the precipitation of protein-protein complex by any of several methods including those utilizing, e.g., an organic solvent such as polyethylene glycol or a salt such as ammonium sulfate. Other suitable separation techniques include, without limitation, the fluorescein antibody magnetizable particle method described in Rattle, et al. (1984) Clin. Chem. 30:1457-1461, and the double antibody magnetic particle separation as described in U.S. Pat. No. 4,659,678.

The methods for linking proteins or their fragments to the various labels have been extensively reported in the literature and do not require detailed discussion here. Many of the techniques involve the use of activated carboxyl groups either through the use of carbodiimide or active esters to form peptide bonds, the formation of thioethers by reaction of a mercapto group with an activated halogen such as chloroacetyl, or an activated olefin such as maleimide, for linkage, or the like. Fusion proteins will also find use in these applications.

Another diagnostic aspect of this invention involves use of oligonucleotide or polynucleotide sequences taken from the sequence of a DX1 protein. These sequences can be used as probes for detecting levels of antigen message in samples from patients suspected of having an abnormal condition, e.g., cancer or developmental problem. The preparation of both RNA and DNA nucleotide sequences, the labeling of the sequences, and the preferred size of the sequences has received ample description and discussion in the literature. Normally an oligonucleotide probe should have at least about 14 nucleotides, usually at least about 18 nucleotides, and the polynucleotide probes may be up to several kilobases. Various labels may be employed, most commonly radionuclides, particularly ^{32}P. However, other techniques may also be employed, such as using biotin modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed which can recognize specific duplexes, including DNA duplexes, RNA duplexes, DNA-RNA hybrid duplexes, or DNA-protein duplexes. The antibodies in turn may be labeled and the assay carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected. The use of probes to the novel anti-sense RNA may be carried out in any conventional techniques such as nucleic acid hybridization, plus and minus screening, recombinational probing, hybrid released translation (HRT), and hybrid arrested translation (HART). This also includes amplification techniques such as polymerase chain reaction (PCR).

Diagnostic kits which also test for the qualitative or quantitative presence of other markers are also contemplated. Diagnosis or prognosis may depend on the combination of multiple indications used as markers. Thus, kits may test for combinations of markers. See, e.g., Viallet, et al. (1989) Progress in Growth Factor Res. 1:89-97.

Methods for Isolating DX1 Specific Binding Partners

The DX1 protein should interact with a ligand based, e.g., upon its similarity in structure and function to other cell markers exhibiting developmental and cell type specificity of expression. Methods to isolate a ligand are made available by the ability to make purified DX1 for screening programs. Soluble or other constructs using the DX1 sequences provided herein will allow for screening or isolation of DX1 specific ligands.

The broad scope of this invention is best understood with reference to the following examples, which are not intended to limit the invention to specific embodiments.

EXAMPLES

General Methods

Some of the standard methods are described or referenced, e.g., in Maniatis, et al. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor Press; Sambrook, et al. (1989) Molecular Cloning: A Laboratory Manual, (2d ed.), vols. 1-3, CSH Press, NY; Ausubel, et al., Biology, Greene Publishing Associates, Brooklyn, NY; or Ausubel, et al. (1987 and Supplements) Current Protocols in Molecular Biology, Greene/Wiley, New York; Innis et al. (eds.)(1990) PCR Protocols: A Guide to Methods and Applications Academic Press, N.Y.

Methods for protein purification include such methods as ammonium sulfate precipitation, column chromatography, electrophoresis, centrifugation, crystallization, and others. See, e.g., Ausubel, et al. (1987 and periodic supplements); Deutscher (1990) "Guide to Protein Purification" in Methods in Enzymology, vol. 182, and other volumes in this series; and manufacturer's literature on use of protein purification products, e.g., Pharmacia, Piscataway, NJ., or Bio-Rad,

Richmond, CA. Combination with recombinant techniques allow fusion to appropriate segments, e.g., to a FLAG sequence or an equivalent which can be fused via a protease-removable sequence. See, e.g., Hochuli (1989) Chemische Industrie 12:69-70; Hochuli (1990) "Purification of Recombinant Proteins with Metal Chelate Absorbent" in Setlow (ed.) Genetic Engineering, Principle and Methods 12:87-98, Plenum Press, N.Y.; and Crowe, et al. (1992) QIAexpress: The High Level Expression & Protein Purification System QUIAGEN, Inc., Chatsworth, CA.

FACS analyses are described in Melamed, et al. (1990) Flow Cytometry and Sorting Wiley-Liss, Inc., New York, NY; Shapiro (1988) Practical Flow Cytometry Liss, New York, NY; and Robinson, et al. (1993) Handbook of Flow Cytometry Methods Wiley-Liss, New York, NY.

Preparation of a Monoclonal antibody.

The DX1 (IgG1) hybridoma was generated by immunizing Balb/c mice with IL-2 activated human peripheral blood NK cells and fusing their splenocytes with the Sp2/0 myeloma cell line and hybridomas were selected for reactivity with human NK cells by indirect immunofluorescence using flow cytometry.

Distribution of DX1 Antigen.

The DX1 antigen is expressed on the cell surface of the majority of CD3-,CD56+ NK cells; however, a subset of NK cells were antigen negative. NK cells can be divided into subsets based on the presence or absence of CD16 and the relative cell surface density of the CD56 antigen. DX1 is expressed on both CD16-,$CD56^{bright}$+ and CD16+,CD56+ NK cells. When NK cell clones were established from adult peripheral blood by directly cloning DX1- and DX1+ NK cells by flow cytometry, re-analysis of the clones after 1 month of culture revealed that NK cell clones initially selected as DX1+ retained expression of the antigen. NK cell clones isolated as DX1- usually remained negative or expressed only low levels of the antigen.

DX1 is also present on a subset of CD3+ T cells, including CD4+ and CD8+ T cells, but is not expressed on B cells, monocytes, or granulocytes. The cell surface density of hNKR-P1A on CD8+ T cells is usually higher than on CD4+ T cells. Both αβ-TcR+ T cells and γδ-TcR+ T cells express DX1. Analysis of peripheral blood from 10 adult donors indicated that DX1 is present on from 73 to 97% (mean 89%) of normal NK cells and from 16 to 47% (mean 24%) of normal T cells.

T cells can be divided into "naive" and "memory" subsets based on expression of several cell surface antigens. The "memory" T cells express higher levels of CD29 and CD95 (fas) than "naive" T cells. Analysis of peripheral blood T cells indicated that DX1 is preferentially expressed on the "memory" T cell subset expressing relatively high levels of CD29 and CD95 antigen. However, there was no evidence for activation of DX1 bearing T cells because these cells did not preferentially express CD25, CD69, or HLA-DR. The ^{125}I labeled DX1 glycoproteins isolated from T and NK cells were essentially indistinguishable when they were analyzed by SDS-PAGE and Non-Equilibrating pH Gel Electrophoresis (NEPHGE), indicating that the anti-NKR-P1A mAb are reacting with the same gene products on these cell types.

DX1 is present on the majority of CD56+ NK cells and a subset of CD3+ T cells present in the fetal liver, indicating that this molecule is expressed early during ontogeny. Similarly, hNKR-P1A was present on the majority of NK cells in cord blood, but was detected only on a minor proportion of T cells in the cord blood (<5% positive). DX1 is expressed on a very minor proportion (<1%) of thymocytes isolated from fetal or postnatal thymus and is present preferentially on a subset of the $CD3^{bright}$ thymocytes.

Biochemical Characterization of DX1.

Anti-DX1 antibody recognizes a disulfide-linked homodimer glycoprotein (MW ~80 kD non-reducing gel; ~40 kD reducing gel) on human T cells and NK cells, based on 2

dimensional (non-reduced/reduced) SDS-PAGE analysis. Cells were labeled with ^{125}I using lactoperoxidase and glucose oxidase as described by Keski-Oja, et al. (1977) Biochem. Biophys. Res. Comm. 74:699-706. Cells were lysed in Tris buffered saline (50 mM Tris, 15 mM NaCl, pH 8.0) containing 1% NP-40 and protease inhibitors (1 mM PMSF and 20 Kallikrein inhibitor units/ml aprotinin) or were lysed in 20 mM triethanolamine/150 mM NaCl buffer (pH 7.8) containing 0.5% digitonin and 0.12% Triton X-100 with protease inhibitors. NKR-P1 antigen was immunoprecipitated from lysates using Pansorbin coated with saturating amounts of rabbit anti-mouse Ig and anti-hNKR-P1A mAb, by the technique described by Lanier, et al. (1987) J. Exp. Med. 165:1076-1094.

^{125}I labeled NKR-P1 glycoproteins were treated with neuraminidase, O-glycanase, and N-glycanase or N-glycosidase F using the conditions recommended by the manufacturers. Two-dimensional NEPHGE gel analysis and two-dimensional diagonal (non-reducing/reducing) gel analysis were performed by the methods described in Lanier, et al. (1987) J. Exp. Med. 165:1076-1094. Digestion of the glycoprotein with N-glycanase to remove N-linked oligosaccharides reduced the apparent molecular weight to ~26 kD. Treatment with neuraminidase slightly decreased the relative mobility of the hNKR-P1A glycoprotein, indicating the presence of sialic acid. O-glycanase failed to affect the mobility of the neuraminidase-treated glycoprotein, suggesting the absence of O-linked sugars.

Isolation of a DNA clone encoding DX1 protein.

RNA was extracted from an IL-2-dependent polyclonal NK cell line using RNAzol™ (Tel-Test, Inc., Friendswood, TX) and cDNA was synthesized using the SUPERSCRIPTASE library kit (BRL, Bethesda, MD). cDNAs were inserted into the Not I (5') and Bst XI (3') sites of the pJFE14 expression vector, a derivative of the SRα expression vector. The NK cDNA library in pJFE14 were transfected into COS7 cells using DEAE-dextran. After 72 hr,

transfected cells were stained with phycoerythrin (PE)-conjugated DX1 mAb, and antigen-positive cells were selected by flow cytometry. DX1+ COS7 cells were lysed in Hirt solution and plasmids were recovered by bacterial transformation, as described by Aruffo and Seed (1987) Proc. Nat'l Acad. Sci. 84:8573. Three rounds of selection by DEAE-dextran transfection were performed and then single colonies were isolated and analyzed.

cDNA inserts were subcloned into the BLUESCRIPT cloning vector (Stratagene, La Jolla, CA) and cDNAs were sequenced by using the dideoxy chain termination technique with the SEQUENASE 2.0 kit (USB, Cleveland, OH). DX1 cDNA were cloned into the pBJ expression vector and murine fibroblast L cells were transfected with hNKR-P1A-pBJ using LIPOFECTIN (BRL, Bethesda, MD) by the technique described by Lanier, et al. (1991) J. Immunol. 146:4421-4426. Stable transfectants were selected by growth in 0.4 mg/ml G418 and L cell lines expressing high levels of DX1 were isolated by flow cytometry.

Nucleotide sequencing revealed a 738 bp cDNA with an open reading frame encoding a 225 amino acid polypeptide with a predicted molecular weight of 26 kD. The predicted polypeptide is a type II membrane protein (i.e., external COOH-terminus) with a 38 amino acid cytoplasmic region, a 29 amino acid transmembrane segment, and a 158 amino acid extracellular domain. Four sites for potential N-linked glycosylation are present in the extracellular domain.

Analysis of the predicted amino acid sequence by the GenBank database (IntelliGenetics, Mountain View, CA) revealed that the DX1 antigen shares 46% identity with the rat NKR-P1 protein and 46-47% identity with the polypeptides encoded by the three mouse NKR-P1 genes, indicating an essentially identical degree of similarity to all of the rodent genes. This degree of identity between the human DX1 and rodent NKR-P1 polypeptides was observed in both the cytoplasmic and extracellular domains. While the cDNA encoding a protein recognized by the DX1 mAb is likely to be the human homolog of the rat and mouse NKR-P1 gene,

it cannot be excluded formally that other rodent molecules with even higher homology may exist.

In another method, oligonucleotides are used to screen a library. In combination with polymerase chain reaction (PCR) techniques, synthetic oligonucleotides in appropriate orientations are used as primers to select correct clones from a library.

Southern and Northern Blot Analysis

Total cellular RNA was isolated using RNAzol™ (TelTest, Inc., Friendswood, TX). Genomic DNA was isolated using the Qiagen Genomic DNA kit (Chatsworth, CA), was digested with restriction enzymes, and was separated by electrophoresis in agarose gels. RNA and DNA were transferred to NYTRAN membranes (Schleicher and Schuell, Keene, NH) and were hybridized with ^{32}P-labeled cDNA probes, generated using random hexamer primers by the method of Feinberg, et al. (1983) Anal. Biochem. 132:6-13.

Northern blot analysis of RNA isolated from a human IL-2-activated polyclonal NK cell line using a ^{32}P labeled DX1 cDNA probe revealed a single broad band of ~0.8 - 1.3 kb. In contrast, in rodents transcripts of several sizes (ranging from 0.5 to 5 kb) have been detected by Northern blot analysis. DX1 transcripts were not detected in RNA isolated from the HPB-ALL T leukemia cell line or the Colo-205 colon carcinoma cell line. The DX1 cDNA was used as a probe to isolate additional bacterial colonies transformed with an IL-2-activated polyclonal NK cell cDNA library.

Southern blots containing DNA prepared from a panel of hamster x human hybrid cell lines were purchased from Bios, Inc. (New Haven, CT) and were used for chromosomal assignment. Radioactivity was detected using a PhosporImager (Molecular Dynamics, Sunnyvale, CA). Southern blot analysis of human genomic DNA using a ^{32}P labeled DX1 cDNA probe revealed a relatively simple restriction enzyme pattern. The DX1 gene was

localized to human chromosome 12. A ^{32}P labeled DX1 probe cross-hybridized with mouse and rat NKR-P1 cDNA in Southern blot analysis, provided that low stringency conditions were used for hybridization and washing.

Biochemical Characterization of the DX1 protein.

A recombinant DX1 construct is prepared which is fused to a useful affinity reagent, e.g., FLAG peptide, useful for purifying the expression product of the construct. See, e.g., Crowe, et al. (1992) QIAexpress: The High Level Expression & Protein Purification System, QUIAGEN, Inc. Chatsworth, CA; and Hopp, et al. (1988) Bio/Technology 6:1204-1210. The sequence allows for efficient affinity purification of the soluble product. Appropriate secretion or processing sites may also be engineered into the construct by standard methods. Purification is achieved by use of affinity purification, e.g., antibodies against the antigen, or by standard protein purification methods. Typically, the affinity reagents or purification procedures can be performed using recombinant receptor.

Preparation of antibodies specific for DX1

Synthetic peptides, purified proteins, or fragements, are presented to an immune system to generate monoclonal or polyclonal antibodies. See, e.g., Coligan (1991) Current Protocols in Immunology,Wiley/Greene; and Harlow and Lane (1989) Antibodies: A Laboratory Manual, Cold Spring Harbor Press. In certain circumstances, a full length protein is preferred, and in others, particular segments, e.g., transmemebrane or extracellular, are preferred. In appropriate situations, the binding reagent is either labeled as described above, e.g., fluorescence or otherwise, or immobilized to a substrate for panning methods.

Purification of the DX1 protein

The DX1 protein is isolated by a combination of affinity chromatography using the DX1 specific binding compositions,

e.g., antibody, as a specific binding reagent in combination with protein purification techniques allowing separation from other proteins and contaminants. Similar techniques using human cell assays and human cell sources are applied to isolate a human antigen.

The DX1 is used for screening of an expression library made from a cell line which expresses a DX1 binding protein, e.g., a ligand. Standard staining techniques are used to detect or sort intracellular or surface expressed ligand, or surface expressing transformed cells are screened by panning. Screening of intracellular expression is performed by various staining or immunofluorescence procedures. See also McMahan, et al. (1991) EMBO J. 10:2821-2832.

For example, on day 0, precoat 2-chamber PERMANOX slides with 1 ml per chamber of fibronectin, 10 ng/ml in PBS, for 30 min at room temperature. Rinse once with PBS. Then plate COS cells at 2-3 x 10^5 cells per chamber in 1.5 ml of growth media. Incubate overnight at 37° C.

On day 1 for each sample, prepare 0.5 ml of a solution of 66 µg/ml DEAE-Dextran, 66 µM chloroquine, and 4 µg DNA in serum free DME. For each set, a positive control is prepared, e.g., of huIL-10-FLAG cDNA at 1 and 1/200 dilution, and a negative mock. Rinse cells with serum free DME. Add the DNA solution and incubate 5 hr at 37° C. Remove the medium and add 0.5 ml 10% DMSO in DME for 2.5 min. Remove and wash once with DME. Add 1.5 ml growth medium and incubate overnight.

On day 2, change the medium. On days 3 or 4, the cells are fixed and stained. Rinse the cells twice with Hank's Buffered Saline Solution (HBSS) and fix in 4% paraformaldehyde (PFA)/glucose for 5 min. Wash 3X with HBSS. The slides may be stored at -80° C after all liquid is removed. For each chamber, 0.5 ml incubations are performed as follows. Add HBSS/saponin (0.1%) with 32 µl/ml of 1M NaN_3 for 20 min. Cells are then washed with HBSS/saponin 1X.

Soluble antibody is added to cells and incubate for 30 min. Wash cells twice with HBSS/saponin. Add second antibody, e.g., VECTOR anti-mouse antibody, at 1/200 dilution, and incubate for 30 min. Prepare ELISA solution, e.g., VECTOR ELITE ABC horseradish peroxidase solution, and preincubate for 30 min. Use, e.g., 1 drop of solution A (avidin) and 1 drop solution B (biotin) per 2.5 ml HBSS/saponin. Wash cells twice with HBSS/saponin. Add ABC HRP solution and incubate for 30 min. Wash the cells twice with HBSS, the second wash for 2 min, which closes the cells. Then add VECTOR diaminobenzoic acid (DAB) for 5 to 10 min. Use 2 drops of buffer plus 4 drops DAB plus 2 drops of H_2O_2 per 5 ml of glass distilled water. Carefully remove chamber and rinse slide in water. Air dry for a few minutes, then add 1 drop of CRYSTAL MOUNT and a cover slip. Bake for 5 min at 85-90° C.

Alternatively, the DX1 proteins are used to affinity purify or sort out cells expressing the ligand. See, e.g., Sambrook, et al. or Ausubel, et al.

Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Schering Corp.

(ii) TITLE OF INVENTION: PURIFIED MAMMALIAN NK ANTIGENS AND RELATED REAGENTS

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Schering Corp.
(B) STREET: One Giralda Farms
(C) CITY: Madison
(D) STATE: New Jersey
(E) COUNTRY: USA
(F) ZIP: 07940

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Apple Macintosh
(C) OPERATING SYSTEM: Macintosh 7.1
(D) SOFTWARE: Microsoft Word 5.1a

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Lunn, Paul G.
(B) REGISTRATION NUMBER: 32,743
(C) REFERENCE/DOCKET NUMBER: DX0397K

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 201-822-7255
(B) TELEFAX: 201-822-7039

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 738 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 61..738

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AAAGCAGAAT TGAGAGTTTG TTCTTACACA CAAGTTTAAT GCCACCTTCC TCTGTCTGCC      60

ATG GAC CAA CAA GCA ATA TAT GCT GAG TTA AAC TTA CCC ACA GAC TCA      108
Met Asp Gln Gln Ala Ile Tyr Ala Glu Leu Asn Leu Pro Thr Asp Ser
 1               5                  10                  15

GGC CCA GAA AGT TCT TCA CCT TCA TCT CTT CCT CGG GAT GTC TGT CAG      156
Gly Pro Glu Ser Ser Ser Pro Ser Ser Leu Pro Arg Asp Val Cys Gln
            20                  25                  30

GGT TCA CCT TGG CAT CAA TTT GCC CTG AAA CTT AGC TGT GCT GGG ATT      204
Gly Ser Pro Trp His Gln Phe Ala Leu Lys Leu Ser Cys Ala Gly Ile
        35                  40                  45

ATT CTC CTT GTC TTG GTT GTT ACT GGG TTG AGT GTT TCA GTG ACA TCC      252
Ile Leu Leu Val Leu Val Val Thr Gly Leu Ser Val Ser Val Thr Ser
    50                  55                  60

TTA ATA CAG AAA TCA TCA ATA GAA AAA TGC AGT GTG GAC ATT CAA CAG      300
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65                  70                  75                  80

AGC AGG AAT AAA ACA ACA GAG AGA CCG GGT CTC TTA AAC TGC CCA ATA      348
Ser Arg Asn Lys Thr Thr Glu Arg Pro Gly Leu Leu Asn Cys Pro Ile
                85                  90                  95

TAT TGG CAG CAA CTC CGA GAG AAA TGC TTG TTA TTT TCT CAC ACT GTC      396
Tyr Trp Gln Gln Leu Arg Glu Lys Cys Leu Leu Phe Ser His Thr Val
            100                 105                 110

AAC CCT TGG AAT AAC AGT CTA GCT GAT TGT TCC ACC AAA GAA TCC AGC      444
Asn Pro Trp Asn Asn Ser Leu Ala Asp Cys Ser Thr Lys Glu Ser Ser
        115                 120                 125

CTG CTG CTT ATT CGA GAT AAG GAT GAA TTG ATA CAC ACA CAG AAC CTG      492
Leu Leu Leu Ile Arg Asp Lys Asp Glu Leu Ile His Thr Gln Asn Leu
    130                 135                 140

ATA CGT GAC AAA GCA ATT CTG TTT TGG ATT GGA TTA AAT TTT TCA TTA      540
Ile Arg Asp Lys Ala Ile Leu Phe Trp Ile Gly Leu Asn Phe Ser Leu
145                 150                 155                 160

TCA GAA AAG AAC TGG AAG TGG ATA AAC GGC TCT TTT TTA AAT TCT AAT      588
Ser Glu Lys Asn Trp Lys Trp Ile Asn Gly Ser Phe Leu Asn Ser Asn
                165                 170                 175

GAC TTA GAA ATT AGA GGT GAT GCT AAA GAA AAC AGC TGT ATT TCC ATC      636
Asp Leu Glu Ile Arg Gly Asp Ala Lys Glu Asn Ser Cys Ile Ser Ile
            180                 185                 190
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TCA CAG ACA TCT GTG TAT TCT GAG TAC TGT AGT ACA GAA ATC AGA TGG      684
Ser Gln Thr Ser Val Tyr Ser Glu Tyr Cys Ser Thr Glu Ile Arg Trp
        195                 200                 205

ATC TGC CAA AAA GAA CTA ACA CCT GTG AGA AAT AAA GTG TAT CCT GAC      732
Ile Cys Gln Lys Glu Leu Thr Pro Val Arg Asn Lys Val Tyr Pro Asp
    210                 215                 220

TCT TGA                                                              738
Ser
225
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 225 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Asp Gln Gln Ala Ile Tyr Ala Glu Leu Asn Leu Pro Thr Asp Ser
 1               5                  10                  15

Gly Pro Glu Ser Ser Ser Pro Ser Ser Leu Pro Arg Asp Val Cys Gln
            20                  25                  30

Gly Ser Pro Trp His Gln Phe Ala Leu Lys Leu Ser Cys Ala Gly Ile
        35                  40                  45

Ile Leu Leu Val Leu Val Val Thr Gly Leu Ser Val Ser Val Thr Ser
    50                  55                  60

Leu Ile Gln Lys Ser Ser Ile Glu Lys Cys Ser Val Asp Ile Gln Gln
65                  70                  75                  80

Ser Arg Asn Lys Thr Thr Glu Arg Pro Gly Leu Leu Asn Cys Pro Ile
                85                  90                  95

Tyr Trp Gln Gln Leu Arg Glu Lys Cys Leu Leu Phe Ser His Thr Val
            100                 105                 110

Asn Pro Trp Asn Asn Ser Leu Ala Asp Cys Ser Thr Lys Glu Ser Ser
        115                 120                 125

Leu Leu Leu Ile Arg Asp Lys Asp Glu Leu Ile His Thr Gln Asn Leu
    130                 135                 140

Ile Arg Asp Lys Ala Ile Leu Phe Trp Ile Gly Leu Asn Phe Ser Leu
145                 150                 155                 160

Ser Glu Lys Asn Trp Lys Trp Ile Asn Gly Ser Phe Leu Asn Ser Asn
                165                 170                 175
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Asp Leu Glu Ile Arg Gly Asp Ala Lys Glu Asn Ser Cys Ile Ser Ile
180 185 190

Ser Gln Thr Ser Val Tyr Ser Glu Tyr Cys Ser Thr Glu Ile Arg Trp
195 200 205

Ile Cys Gln Lys Glu Leu Thr Pro Val Arg Asn Lys Val Tyr Pro Asp
210 215 220

Ser
225

WHAT IS CLAIMED IS:

1. A nucleic acid encoding a DX1 protein or fragment thereof.

2. The nucleic acid of claim 1 wherein the nucleic acid contains SEQ ID NO: 1 or a subsequence thereof.

3. A DX1 protein, a fragement of a DX1 protein or a fusion protein containing a DX1 protein sequence.

4. The protein of claim 3 wherin the DX1 protein or fragment is of primate origin.

5. A DX1 protein or fragment thereof whichexhibits a post-translational modification pattern distinct from a natural DX1 protein.

6. A DX1 protein or fragment containig all of a portion of SEQ ID NO:2.

7. An antibody raised against a primate DX1 protein or against a fragment thereof.

8. The antibody of claim 7 wherein the antibody is raised against a peptide sequence of SEQ ID NO:2.

9. An antibody of claims 7 or 8 wherein the antibody is a monoclonal antibody.

10. An antibody of claims 7, 8 or 9 wherein the anibody is labeled.

12. A method of screening a sample for a ligand for DX1 comprising the steps of producing a purified or recombinant DX1 protein, and screening in said sample for a specific binding of said ligand to said DX1 protein.

13. A method of modulating physiology or development of a cell comprising contacting said cell with an agonist or antagonist of a DX1 protein.

14. A method of Claim 13, wherein said antagonist is an antibody against a primate DX1 protein.

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 94/07587

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/705 C07K16/28 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CURRENT OPINION IN IMMUNOLOGY, vol.5, no.1, February 1993 pages 67 - 73 YOKOYAMA, W M.; 'Recognition structures on natural killer cells' see the whole document ---	1-14
A	GLYCOBIOLOGY, vol.3, no.1, February 1993 pages 9 - 14 CHAMBERS W H;ADAMKIEWICZ T;HOUCHINS J P; 'TYPE II INTEGRAL MEMBRANE PROTEINS WITH CHARACTERISTICS OF C-TYPE ANIMAL LECTINS EXPRESSED BY NATURAL KILLER NK CELLS GLYCOBIOLOGY.' -----	1-14

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 October 1994	08 -11- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Nauche, S

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US 94/07587

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Altough claims 13-14 are directed to a method of the human/ animal body as well as diagnostic methods, as far as applied invivo, (rule 39.1 (iv) PCT) the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.